Modeling the Inactivation and Possible Regrowth of *Salmonella enterica*
Treated with Chlorophyllin-Chitosan and Visible Light

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SUMMARY

The study is focused on predictive modelling of inactivation of *Salmonella enterica* after treatment with chlorophyllin-chitosan (Chl-CHS) and visible light. *Salmonella* cells were incubated with Chl-CHS (1.5x10^-5 M 0.001 Chl 0.1 % CHS) for different times (5-60 min) and then illuminated with visible light (λ=405 nm, 38 J/cm^2). Inactivation curves as well as post-treatment regrowth curves were build based on microbiological viability tests and data were fitted to 10 inactivation and two regrowth models. Photoactivated Chl-CHS reduced *Salmonella* population which were unable to regrowth. Weibull and Baranyi models were the best to describe the inactivation and regrowth kinetics respectively. In conclusion, data from the kinetic analysis and predictive modeling confirmed that photoactivated Chl-CHS is a promising non-thermal approach for inactivation of Gram-negative pathogens, since no bacterial regrowth after treatment has been predicted.

Key words: photosensitization, microbial modeling, chlorophyllin, chitosan, *Salmonella*, microbial inactivation

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INTRODUCTION

According to the World Health Organization (WHO) the incidence of foodborne diseases is a drastically growing public health problem in the world (1). Likewise, the Centers for Disease Control and Prevention (CDC) reported 48 million illnesses, 128,000 hospitalizations, and 3,000 deaths every year due to foodborne illness caused by pathogenic microorganisms (2). Fresh produce became the second leading cause of foodborne illnesses, which poses a $77.7 billion economic burden in the US annually (3). Salmonella is one of the most important foodborne pathogens. In 2013, Salmonella affected more than 1 million people in the USA, with 19,336 hospitalizations and 378 deaths, and associated costs over 3.7 billion US dollars (4). A recent study, elaborated with outbreak data from 2007 to 2011 in the European Union, has identified Salmonella as the microorganism most often linked to human cases of foodborne illnesses in ready-to-eat unprocessed foods of non-animal origin (5). Consequently, the control of foodborne infections remains a global problem with significant social and economic impact (6). In this context, innovative, effective, not chemical and environmentally friendly antimicrobial technologies are highly requested.

To this end, a modern biophotonic technology based on photosensitization and successfully used to cure cancer and infectious diseases (photodynamic therapy) (7) is under study for the decontamination of fresh produce and food-related surfaces (8-10). It is interesting to note, that chlorophyllin (Chl), which is a food colorant authorized for use in the food industry in the European Union (E140ii) in accordance with Annex II to Regulation (EC) No 1333/2008 (11) and in the USA according to regulation 21CFR73.125 (12) can act as very effective photosensitizer (13). This nonthermal treatment is based on the combined action of photosensitizer, light and oxygen, which eventually produces reactive oxygen species and triggers the death of all microorganisms that interact with photosensitizer (13). At molecular level, photosensitized Chl inactivates bacteria by generation of \( \cdot O_2 \), causing oxidative stress to bacteria and increasing cell membrane permeability, which occurs while bacterial cell upregulates genes responsible for detoxification of reactive oxygen species and downregulates genes responsible for inhibition of oxidative respiration, cell division and metabolism (14).

The main advantage of photosensitization is its high efficiency against a wide range of microorganisms: Gram-positive and Gram-negative bacteria, their vegetative forms and spores, as well as fungi and yeasts and is as effective as high power pulsed UV light (15). Moreover, this treatment is environmentally friendly, cost-effective, saving water and energy (16). Though, there is one important disadvantage of this antimicrobial treatment: susceptibility
of Gram-negative bacteria to photosensitization using negatively charged photosensitizer Chl is lower than that of Gram-positive bacteria (17, 18). This new challenge prompted us “to turn” to the hurdle technologies, i.e. to combine Chl-based photosensitization with other antimicrobials (14).

Chitosan (CHS) is a food additive derived from chitin also approved in the USA and European Union (19). It can form a Chl-DHS complex by interaction between its positively charged \( \text{NH}_3^+ \) group and the negatively charged Chl \( \text{COO}^- \) group, which can be excited at 405 nm (8).

In order to compare quantitatively the efficiencies of different antimicrobial treatments, modelling of the inactivation of bacteria and their re-growth dynamics is most reliable. While modelling of bacterial growth after treatment is relatively easy (since growth curves have a single exponential or sigmoidal shape), modelling of inactivation curves is not straightforward due to the wide variety of shapes that can be found, ranging from a simple log-linear shape to complex multiphasic ones. The latter has given place to several models as summarized by Geeraerd et al. (20). Usually, the selection of the best fitting model is based on statistical fitting. The goal of this research was to evaluate using mathematical models the inactivation of Salmonella enterica treated by photoactivated Chl-DHS and to predict the possible regrowth of this bacterium after treatment.

MATERIALS AND METHODS

Experiments

All experimental conditions to inactivate bacteria and data have been described in Buchovec et al. (9, 14). In brief, experiments were carried out with Salmonella enterica serovar Typhimurium strain DS88 [SL5676 SmR (pLM32)] resistant to tetracycline. Non-copperized chlorophyll sodium salt (Roth, Karlsruhe, Germany) and low molecular weight chitosan (Aldrich, Saint Louis, USA) were used for complex preparation. 1.5x10^-5 M 0.001 Chl and 0.1 \% CHS complex in 0.9 \% NaCl were used for experiments. Light inactivation (38 J/cm²) was carried out by an own-made light device equipped (Fig. 1) with 60 light emitting diodes (LEDs), InGaN LED array (LED Engine, San Jose, USA; Inc. LZ1-00UA00) with peak emission at 405 nm as described by Buchovec et al. (9). Thus, microbial inactivation experiments consisted in the incubation of Salmonella with Chl-DHS complex for 5, 10, 15, 30, 45 or 60 min and subsequent illumination. Treatments were carried out on 150 µL of samples placed in sterile flat bottom wells at room temperature and pH=7.2. Treatments were: 1) control (no sensitizer, no light) (CTRL), 2) chitosan with no light (CHS), 3) photosensitized chlorophyllin (Chl+hv), 4) chlorophyllin-chitosan with no light (Chl-CHS) and 5) photosensitized chlorophyllin-chitosan...
(Chl-CHS+hv). Then, *S. enterica* survival populations were enumerated by plate counting. Microbial regrowth was determined in treatments Chl–CHS and Chl–CHS+hv (and control) by measuring absorbance at 540 nm during incubation in the dark at 37 °C. Experiments were repeated four times.

--- Figure 1 here ---

**Modelling of Salmonella inactivation by photoactivated chlorophyllin-chitosan**

Inactivation kinetics were analyzed using GinaFit add-in tool for Excel (20). Data were fitted to all 10 different kinetic models available in this software; only the three models that show the best fit to data have been included in this report.

The log-linear model read as follows:

$$\log N = \log N_0 - \frac{k_{\text{max}} \cdot t}{\ln 10}$$  \hspace{1cm} /1/

where $N$ represents the number of microorganisms that survived the different treatments (CFU/mL), $N_0$ represents the initial population (CFU/mL), $k_{\text{max}}$ is the specific inactivation rate (1/min) and $t$ the treatment time (min).

The Weibull model reads as follows:

$$\log N = \log N_0 - \left(\frac{t}{\delta}\right)^{\rho}$$ \hspace{1cm} /2/

where $\delta$ is the scale parameter (min) and the $\rho$ parameter characterizes the shape of the curves (dimensionless) (when $\rho$ is less than 1, the curve shows an ascending concavity; when $\rho$ is greater than 1, the curve had a concavity downwards).

The log-linear and tail model (20) read as follows:

$$\log N = \log\left(10^{\log N_0} - 1\right)\log N_{\text{res}} + \log(e^{-k_{\text{max}} \cdot t} + 10^{\log N_{\text{res}}})$$ \hspace{1cm} /3/

where $N_{\text{res}}$ (CFU/mL) is the residual population density.

To evaluate the degree of adjustment of the model, the mean square error ($RMSE$) and the coefficient of determination $R^2$ were used. High $R^2$ and low $RMSE$ values indicate a better fit of the model. The $RMSE$ is determined by the following equation:

$$RMSE = \sqrt{\frac{\sum(P-O)^2}{n-p}}$$ \hspace{1cm} /4/

where $P$ is the predicted value, $O$ the observed value, $n$ is the number of observations and $p$ is the number of parameters to be estimated.

Weibull model for microbial inactivation was validated with three sets of data from additional tests. Deviations from the predicted values were analyzed and the $RMSE^*$ value was
calculated as measurement of the performance of the model (21). RMSE* values were obtained using equation 4 with p=0.

*Modelling of Salmonella regrowth after treatment with photoactivated chlorophyllin-chitosan*

Re-growth data were fitted to different models using the DMFit shareware package for Excel (22). The equation of Baranyi and Roberts (1994) is as follows:

\[
\ln N = \ln N_{max} + \ln\left(\frac{-1 + e^{\mu_{max} \lambda + e^{\mu_{max} t}}}{-1 + e^{\mu_{max} \lambda + e^{\mu_{max} t}} + \ln N_{max} - \ln N_0}\right) \tag{5}
\]

where \(N_0\) (absorbance) is the lower asymptotic value and approximately equal to the initial population density, \(N_{max}\) (absorbance) is the upper asymptotic value and approximately equal to the maximal population density, \(\mu_{max}\) (absorbance 1/h) is the maximum growth rate and \(\lambda\) (h) is the latency time.

The equation of Gompertz (23) states that:

\[
\ln \frac{N}{N_0} = A \cdot e^{-\exp\left(\frac{\mu_{max} - \epsilon}{A} (\lambda - t) + 1\right)} \tag{6}
\]

where \(A\) (absorbance) is the maximum size of the microbial population.

**RESULTS AND DISCUSSION**

*Modelling the inactivation of Salmonella enterica by photoactivated chlorophyllin-chitosan complex.*

The inactivation of *S. enterica* by a photoactivated Chl-CHS (Chl-CHS+hv) complex *in vitro* was investigated using different incubation times (5-60 min) and the potential antimicrobial effects of individual experimental factors were also investigated in order to assess if the observed inactivation requires the combination of those factors.

Ten models were applied for analysis of parameters of microbial inactivation and results for the three best performers are shown in Table 1. Only Chl+hv and Chl-CHS+hv treatments caused significant inactivation. The slope of the log-linear model for CHS and Chl-CHS treatments demonstrated that no inactivation was achieved by these treatments. Comparing the different models for treatment Chl-CHS+hv, it can be seen that the Weibull model was the one that fitted the best since its RMSE value was the closest to 0 and its R² was the closest to 1 (20). In case of Chl+hv treatment, both, Weibull and log-linear + tail models yielded similar results, the first was selected to make it the same for both treatments and allow comparing parameters. The scale parameter for the Chl-CHS+hv treatment was four times lower than that of the Chl+hv treatment; which indicated that this treatment has higher inactivation efficacy.

The good fitting of the Weibull distribution to data is an excellent indicator that the kinetics of *Salmonella* inactivation by photosensitized Chl-CHS is a consequence of the progressive
inactivation of *Salmonella* cells having different photosensitization resistances (24). This outcome is not surprising given the nature of our experiments. It should be noticed that in the current case, the microbial inactivation has been described not as function of photosensitization time but as function of the time of incubation of *Salmonella* cells with the Chl-CHS complex. Therefore, a progress in the inactivation curve should mean more accumulation of Chl-CHS complex on the cell (14) and consequently, more sensibility to light inactivation. This result can be useful for selecting photosensitizing concentrations to be used in further studies of other parameters, such as illumination time. It should be noticed that the validity of this as well as other models is restricted to the microorganism under study. Other microorganisms can render curves that can be fitted by other models or even by the same model but with other parameter values. Further studies about the effect of this inactivation method on other microorganisms and in real foods would be beneficial for the overall assessing of the efficacy of this method for achieving food safety goals.

The RMSE* index was used as measure of performance of the Weibull model to fit the inactivation data (Table 2). RMSE* for Chl+hv and Chl-CHS+hv treatments were low and close to those found when the models were built, indicating good performance. A point-by-point analysis for the case of the Chl+hv treatment shows that the model fails-dangerous from the 30 min of treatment on, however, this treatment yielded very low inactivation is unlikely to be used. The Chl+hv treatment was indeed useful in the frame of the current research only to test the potential enhancement of lethality of a Chl+hv treatment when Chl is complexed with CHS. For the case of the Chl-CHS+hv treatment, results show that the model fails-safe (overestimates) the initial population level and the middle part of the inactivation curve and fails-dangerous (underestimates) the rest of the curve. The underestimation observed at 60 min treatment time has no practical relevance since at that time the counts have already fallen to zero. Further studies should validate the model in real food systems.

Predictive modeling of regrowth of inactivated *Salmonella enterica* population
While a high level of microbial inactivation can render a food safe and stable, surviving microorganisms can grow during food storage and can threat their safety and stability. This is the reason why it is not only important to evaluate microbial inactivation but also the regrowth of the survival population. After the inactivation by photoactivated Chl-CHS tests, the dynamics of regrowth of *S. enterica* populations was followed (Fig. 2). This included Chl+hv and Chl-
CHS+hv because other treatments did not lead to inactivation. The regrowth of a non-treated S. enterica population was also evaluated for comparison (control). It can be observed that Chl-CHS+hv treatment produced a damage to S. enterica population that made it impossible to regrowth during the first 15 hours’ post-treatment under the culturing conditions used in this research. No further measurements were registered since the growth curves corresponding to the other two treatments reached the stationary phase at that time. In contrast, S. enterica population treated with Chl+hv can regrow in a similar way as control bacteria. The absence of regrowth can indicate that either no bacterial cells survived the photosensitization treatment or survivors were sub-lethally damaged and unable to grow. Sub-lethal injury has been reported for Escherichia coli and Staphylococcus aureus cells subjected to photosensitization with curcumin and blue light (25).

Two growth models, the Baranyi and Roberts model and the Gompertz model, were tested for fitting regrowth curves. The Baranyi and Roberts (1994) model (22) yielded a good fit for those populations that were able to grow. The control Salmonella grows with a maximal growth rate (μ) of 0.46 abs/h, and a lag phase of 1.69 hours (Table 3). When these values were statistically compared with those obtained for the Chl+hv treatment using the t Student test, a p=0.2675 was found for the maximal growth rate, therefore, both populations grow at the same rate. In contrast, when the same test was used to compare lag phase duration, a p=0.002 was obtained, which indicated that the Chl+hv treatment causes a post-treatment damage to S. enterica strong enough to stop its growth. When the growth parameters of the Chl-CHS+hv treatment were compared with those of the control, the maximal growth rate of the latter was 291 times higher, indeed, Chl-CHS+hv treated populations do not pass the lag phase. Similar results have been reported for Chl+hv treatment of S. enterica when the light source was provided by a pulsed light system, which provides high-intensity broad-spectrum light (26). However, while pulsed light is known to require short exposure times, it is still an expensive technology.

Determining regrowth potential is important for prognosis of the safety of foods after microbial inactivation. The inactivation of foodborne pathogens is seldom complete and is sometimes overestimated when viable but non culturable forms are induced. Therefore, the regrowth potential should be assessed. The absence of regrowth in photoactivated Chl-CHS treatment contrast with the well-known recovery that bacteria can undergo after UV-C light treatment, both in dark or illuminated conditions (27). Regrowth has also been observed to occur after the
application of other non-thermal methods such as high hydrostatic pressure (28) and pulsed light (29). Besides the effect of this method on the inactivation of other microorganisms, further studies such as its application to foods and potential effects on food quality and shelf-life are advised.

CONCLUSION

The inactivation kinetics of Gram-negative food pathogen *Salmonella enterica* by a chlorophyllin-chitosan (Chl-CHS) complex activated with LED-based light at $\lambda=405$ nm and its regrowth were modelled. Photoactivated Chl-CHS treatment was able to decrease *S. enterica* counts and no regrowth was observed after 15 hours of incubation. Weibull and Baranyi models were the best to describe the inactivation and regrowth kinetics respectively. Validation show the good performance of the Weibull to describe the inactivation kinetics. Further studies should validate the models in real food systems. The high inactivation efficacy of photoactivated Chl-CHS treatment and the lack of recovery of populations after this treatment unlocks its huge potential as promising nonthermal and not-chemical approach to control food pathogens on different surfaces.

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CONFLICT OF INTEREST

The authors declare that no conflict of interest exists.

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Table 1. Microbial kinetics modelling.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Parameters</th>
<th>Log-linear</th>
<th>Weibull</th>
<th>Log-linear+tail</th>
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<tr>
<td></td>
<td>RMSE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chl-CHS+hv</td>
<td></td>
<td>1.3203</td>
<td>0.3633</td>
<td>0.7436</td>
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<tr>
<td></td>
<td>R²</td>
<td>0.6996</td>
<td>0.9773</td>
<td>0.9047</td>
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<tr>
<td></td>
<td>Log N₀/(log CFU/mL)</td>
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<td>6.96±0.18</td>
<td>6.21±0.32</td>
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<td></td>
<td>Kₘₐₓ/(1/min)</td>
<td>0.21±0.03</td>
<td>0.72±0.08</td>
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<tr>
<td></td>
<td>δ/min</td>
<td>0.04±0.03</td>
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<td></td>
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<tr>
<td></td>
<td>p/(-)</td>
<td>0.27±0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Log Nₘₑₜ/(log CFU/mL)</td>
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<td>0.56±0.21</td>
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</tr>
<tr>
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<td></td>
<td>0.1326</td>
<td>0.1297</td>
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<td></td>
<td>R²</td>
<td>0.7578</td>
<td>0.7683</td>
<td>0.7797</td>
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<td></td>
<td>Log N₀/(log CFU/mL)</td>
<td>6.78±0.05</td>
<td>6.83±0.06</td>
<td>6.84±0.06</td>
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<td>Kₘₐₓ/(1/min)</td>
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<td>δ/min</td>
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<td>p/(-)</td>
<td>0.69±0.20</td>
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<td>Log Nₘₑₜ</td>
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<td>6.08±0.16</td>
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<tr>
<td>CHS</td>
<td></td>
<td>0.1271</td>
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<td></td>
<td>R²</td>
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<td>Log N₀/(log CFU/mL)</td>
<td>6.77±0.04</td>
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<td>Kₘₐₓ/(1/min)</td>
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<td>Chl-CHS</td>
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<td>Log N₀/(log CFU/mL)</td>
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<td>Kₘₐₓ/(1/min)</td>
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Table 2. Validation of the Weibull model for the inactivation of *S. enterica* in phosphate buffered saline by photoactivated Chlorophyllin and photoactivated Chlorophyllin-Chitosan.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Chl+hv</th>
<th>Chl-CHS+hv</th>
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<tr>
<td></td>
<td>Observed</td>
<td>Predicted</td>
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<tr>
<td>0</td>
<td>6.76</td>
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</tr>
<tr>
<td>0</td>
<td>6.76</td>
<td>6.83</td>
</tr>
<tr>
<td>0</td>
<td>6.72</td>
<td>6.83</td>
</tr>
<tr>
<td>30</td>
<td>6.72</td>
<td>6.41</td>
</tr>
<tr>
<td>30</td>
<td>6.77</td>
<td>6.41</td>
</tr>
<tr>
<td>30</td>
<td>6.67</td>
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<tr>
<td>60</td>
<td>6.72</td>
<td>6.16</td>
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</tbody>
</table>

RMSE* 0.3424 0.4065

Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

Table 3. Microbial regrowth modeling

<table>
<thead>
<tr>
<th>Model</th>
<th>Treatment</th>
<th>Maximal growth rate ($\mu$/abs h$^{-1}$)</th>
<th>Lag phase/h</th>
<th>R$^2$</th>
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<tbody>
<tr>
<td>Baranyi and Roberts</td>
<td>Control</td>
<td>0.460±0.023</td>
<td>1.688±0.105</td>
<td>0.9910</td>
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<td>Chl+hv</td>
<td>0.478±0.021</td>
<td>2.32±0.122</td>
<td>0.9856</td>
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<tr>
<td></td>
<td>Chl-CHS+hv</td>
<td>0.002±0.000</td>
<td>15.000</td>
<td>0.0191</td>
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<tr>
<td>Gompertz</td>
<td>Control</td>
<td>0.551±0.028</td>
<td>2.306±0.068</td>
<td>0.9920</td>
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<tr>
<td></td>
<td>Chl+hv</td>
<td>0.560±0.015</td>
<td>2.803±0.119</td>
<td>0.9838</td>
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<tr>
<td></td>
<td>Chl-CHS+hv</td>
<td>0.008±0.004</td>
<td>0.109±0.188</td>
<td>0.0521</td>
</tr>
</tbody>
</table>

Control=no sensitizer and no light. Chl+hv=Photoactivated Chlorophyllin. Chl-CHS+hv=Photoactivated Chlorophyllin-chitosan complex. Estimated parameters are expressed as mean ± standard error.
Figure 1: LED -based light source with illumination of the sample from both sides, time and intensity control.

Figure 2: Growth curve of *S. enterica* after inactivation by photosensitized chlorophyllin-chitosan complex. Control: no treatment. Chl+hv: photosensitized chlorophyllin. Chl-CHS+hv: photosensitized chlorophyllin-chitosan complex. Curves are fitted to Baranyi and Roberts model (22). Bars represent standard deviation. Data from references (9, 14).